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The interaction between anti-PF4 antibodies and anticoagulants in vaccine-induced thrombotic thrombocytopenia

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Abstract:
Life threatening thrombotic events at unusual sites have been reported after vector-based vaccinations against SARS-CoV-2. This phenomenon is now termed as vaccine-induced immune thrombotic thrombocytopenia (VITT). Pathophysiology of VITT is similar to that of heparin-induced thrombocytopenia (HIT), and associated with platelet-activating antibodies against platelet factor 4 (PF4). Therefore, current guidelines suggest non-heparin anticoagulants to treat VITT patients. In this study, we investigated the interactions of heparin, danaparoid, fondaparinux and argatroban with VITT-Ab/PF4 complexes using *ex vivo* model for thrombus formation as well as *in vitro* assays to analyze antibody binding and platelet activation. We found that IgGs from VITT patients induce increased adherent platelets/thrombus formation in comparison to IgGs from healthy controls. In this ex *vivo* flow-based model, the procoagulant activity of VITT IgGs was effectively inhibited with danaparoid, argatroban but also by heparin. Interestingly, heparin and danaparoid not only inhibited IgG binding to PF4 but were also able to effectively dissociate the preformed PF4/IgG complexes. Fondaparinux reduced the *in vitro* generation of procoagulant platelets and thrombus formation, however it did not affect platelet aggregation. In contrast, argatroban showed no effect on procoagulant platelets and aggregation, but significantly inhibited VITT-mediated thrombus formation. Taken together, our data indicate that negatively charged anticoagulants can disrupt VITT-Ab/PF4 interactions, which might serve as an approach to reduce antibody-mediated complications in VITT. Our results should be confirmed, however, in a clinical setting before a recommendation regarding the selection of anticoagulants in VITT patients could be made.

Conflict of interest: COI declared - see note

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Running head: Anticoagulants in VITT

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Key points

1. Heparin and danaparoid inhibit VITT antibody-mediated thrombus formation, and procoagulant platelet generation.
2. Heparin and danaparoid interfere with the binding of VITT antibodies to PF4, leading to the dissociation of pre-formed VITT-Ab/PF4 complexes.

Abstract

Life threatening thrombotic events at unusual sites have been reported after vector-based vaccinations against SARS-CoV-2. This phenomenon is now termed as vaccine-induced immune thrombotic thrombocytopenia (VITT). Pathophysiology of VITT is similar to that of heparin-induced thrombocytopenia (HIT), and associated with platelet-activating antibodies against platelet factor 4 (PF4). Therefore, current guidelines suggest non-heparin anticoagulants to treat VITT patients. In this study, we investigated the interactions of heparin, danaparoid, fondaparinux and argatroban with VITT-Ab/PF4 complexes using *ex vivo* model for thrombus formation as well as *in vitro* assays to analyze antibody binding and platelet activation. We found that IgGs from VITT patients induce increased adherent platelets/thrombus formation in comparison to IgGs from healthy controls. In this ex *vivo* flow-based model, the procoagulant activity of VITT IgGs was effectively inhibited with danaparoid, argatroban but also by heparin. Interestingly, heparin and danaparoid not only inhibited IgG binding to PF4 but were also able to effectively dissociate the preformed PF4/IgG complexes. Fondaparinux reduced the *in vitro* generation of procoagulant platelets and thrombus formation, however it did not affect platelet aggregation. In contrast, argatroban showed no effect on procoagulant platelets and aggregation, but significantly inhibited VITT-mediated thrombus formation. Taken together, our data indicate that negatively charged anticoagulants can disrupt VITT-Ab/PF4 interactions, which might serve as an approach to reduce antibody-mediated complications in VITT. Our results should be confirmed, however, in a clinical setting before a recommendation regarding the selection of anticoagulants in VITT patients could be made.

Introduction

The ongoing COVID-19 pandemic has led to a significant amount of loss to human lives during last 24 months.[1,2] However, a major breakthrough has been achieved in tackling the pandemic by rapid development, roll out and administration of many vaccines against SARS-CoV-2.[1,2] Although the vaccines are largely effective and considered safe from adverse events, with increasing number of vaccinations across the world, there have been reports of serious illnesses after vaccine administration. Some of the most significant adverse events are reported in people vaccinated with ChAdOx1 nCoV-19 vaccine (Vaxzevria: University of Oxford/AstraZeneca), and collectively termed as vaccine-induced immune thrombotic thrombocytopenia (VITT).[3-6] Patients with VITT develop severe thrombocytopenia and thrombosis 1 to 4 weeks after vaccination, such as the cerebral venous sinus thrombosis (CVST) or splanchnic vein thrombosis (SVT), pulmonary embolism (PE) and deep vein thrombosis (DVT), with a mortality rate of 30%.[7] Serological findings in VITT patients resemble clinical manifestation of spontaneous heparin-induced thrombocytopenia (HIT), which is an immune-mediated thrombotic and thrombocytopenic condition developing even without any previous heparin exposure. Similar to HIT, antibodies directed against platelet factor 4 (PF4) are detected in the sera from patients with VITT.[6-8] These antibodies bind to PF4 and form immune complexes, which in turn activate platelets through Fc gamma receptor IIA (FcγRIIA).[6,9]

A number of guidelines have been issued regarding the diagnosis and treatment of VITT.[10,11] The recommendations are mainly based on the experience on HIT due to the similarities in the pathophysiology of HIT and VITT. To prevent any potential thrombotic incidences because of binding with IgG-PF4 immune complexes, VITT guidelines recommend avoidance of anticoagulation with heparin, and rather to use non-heparin anticoagulants such as dabigatran, fondaparinux and argatroban.[10,11] Although growing evidence suggests that the use of non-heparin anticoagulants is beneficial for VITT patients, their use in

thrombocytopenic patients, especially those with increased risk for occlusive cerebral bleeding remains a clinical challenge in the absence of an efficient antidote.

To our best knowledge, the effect of heparin and non-heparin anticoagulants on the interaction between VITT antibodies and PF4, platelet activation and thrombus formation has not been addressed so far. Awaiting *in vivo* models for VITT, *ex vivo* model could not only improve our understanding to the pathomechanistic events of the disease but also to evaluate therapeutic approaches to prevent thrombosis progression in affected patients. In this study, we systemically analyzed the interactions of heparin, danaparoid, fondaparinux, and argatroban with VITT-IgG/PF4 complexes using microfluidic system as well as several functional and immunoassays to investigate the ability of these anticoagulants to interfere with antibody binding as well as thrombus formation. Since intravenous immune globulin (IVIG) is used to treat VITT patients, it was also included in some experimental settings to analyze any synergistic effects.

Methods

Patient cohort

Patients with suspected VITT were referred to our hospital after vaccination with ChAdOx1 nCoV-19 (AstraZeneca, London, UK). Blood samples obtained from patients with confirmed VITT were included in this study. Laboratory and clinical data were reviewed by three physicians (G.U., K.A., and T.B.). Serological investigation included blood count, D-Dimer, PF4/Hep enzyme immunoassay (EIA) and heparin-induced platelet activation assay (HIPA). The diagnosis of VITT was considered confirmed if IgG antibodies against PF4 were detected in EIA and the modified HIPA revealed positive reaction within 30 min in the presence of PF4 with at least two out of four platelet donors.

Investigations of VITT antibody-mediated thrombus formation

To assess the impact of VITT IgGs, a flow-based *ex vivo* model for platelet adhesion/thrombus formation in whole blood was established utilizing citrated blood which

was recalcified and hirudin. A microfluidic system (BioFlux 200, Fluxion Biosciences, Alameda, USA) was used according to the recommendations of the ISTH (International Society on Thrombosis and Haemostasis) standardization committee for biorheology.[12] Briefly, microfluidic channels were coated with collagen (100 µg/mL, Collagen Horn, Takeda, Linz, Austria) overnight at 4°C and blocked with 2.5% of human serum albumin (HSA, Kedrion, Barga, Italy).

Whole blood samples of healthy individuals of blood group O were collected and allowed to rest for 30 min at room temperature (RT). After splitting the whole blood into aliquots of 200 µL, platelet-rich plasma (PRP) was prepared via centrifugation (20 min, 120g, at RT, no break). IgG fractions were isolated from sera of VITT patients as described in supplemental methods. Afterwards, 45 µL of the supernatant PRP was gently separated and incubated with 5 µL of IgG fraction from VITT or healthy control sera, and incubated for 90 min at RT under rotating conditions. Platelets were fluorescently labelled by adding Calcein-FITC (4 µM, Thermo Fisher Scientific, Waltham, USA) 15 min prior to the end of the incubation period. Subsequently, PRP was gently added back to reconstitute whole blood samples. Where indicated, platelets were incubated with anticoagulants: heparin (unfractionated heparin, 0.1 and 0.5 U/mL), danaparoid (0.8 U/mL), fondaparinux (1 and 8 µg/mL) and argatroban (0.8 and 8 µg/mL).

To assess the impact of IVIG on thrombus formation, microfluidic channels were prepared as described above, and where indicated, IgG treated PRP were co-incubated with IVIG in presence or absence of anticoagulants. Finally, reconstituted whole blood samples were perfused at a shear rate of $250s^{-1}$ (10 dyne) for a maximum of 15 min. Immunofluorescence images were acquired from randomly selected microscopic fields (Olympus IX73, Olympus GmbH, or Zeiss Axio Observer, Karl Zeiss, Germany). Thrombus formation was determined as measuring surface area coverage (SAC) by adherent platelets on collagen. Adherent platelets were quantified as the percent (%SAC) of 5 images via ImageJ (NIH, Bethesda, USA) as previously described.[13]

Assessment of antibody-mediated procoagulant platelet generation and platelet aggregation

The impact of anticoagulants on the ability of VITT sera to induce procoagulant phenotype and platelet aggregation was assessed using flow cytometry and the HIPA assay, respectively, as described previously[6,13]. For more details, see supplemental methods.

Interaction between VITT Abs and anticoagulants in immunoassays

Patients' sera were tested for anti-PF4 antibodies using an in-house PF4 enzyme-linked immunosorbent assay (PF4-EIA). Detailed information is available in supplemental materials. In brief, microtitre plates were coated overnight with PF4 (25 µg/mL), before blocking with 3% bovine serum albumin (BSA). After subsequent washing, plates were incubated with patient sera (1:50 diluted, 1h, RT), followed with further washing, and incubation with 1:1000 diluted peroxidase-conjugated anti-human IgG (RT, 1 h). Substrate tetramethylbenzidine (TMB one) was added and the reaction was stopped with H_2SO_4, Absorbance was measured at 450 nm with a microplate reader.

Inhibition assay

The ability of anticoagulants to inhibit VITT IgG binding to PF4 was analyzed by adding heparin, danaparoid, fondaparinux and argatroban to the patient serum (illustration in Figure 3A) prior to the incubation with PF4 coated plates as previously described.[14] Anticoagulants were used at a concentration of 0.07, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.33, 16.67, and 33.33 U/mL or µg/mL if not indicated otherwise. Vehicle controls were used in same dilution series as a control. IgG antibody binding was determined as described above. Test results were normalized to buffer.

Dissociation assay

IgG/PF4 complex dissociation was determined with a slight modification in PF4-EIA assay (illustration in Figure 3B). VITT-IgG containing sera were first incubated on the PF4 plates for an hour to facilitate the complex formation. This step was followed by incubation of serially

diluted anticoagulants (1h, RT) and remaining bound IgG antibodies were detected as described above. Test results were normalized to buffer.

Assessment of IgG binding to platelet surface

IgG binding to platelets after incubation with sera from VITT patients was assessed using flow cytometry (illustration in Figure 3C and 3D) in presence or absence of exogenous PF4. In brief, cell suspensions were preincubated with PF4 (25 µg/mL) prior to incubation of anticoagulants and patients' sera for 1.5 h at RT with gentle rotation. FITC-conjugated mouse anti-human IgG (BD Biosciences) was added and directly analyzed by flow cytometry (FC). IgG binding was expressed as fold increase normalized to healthy controls.

Binding kinetics of antibodies using biolayer interferometry (BLI)

Purified PF4 (ChromaTec, Greifswald, Germany) was biotinylated with Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific, Waltham, USA) in 5 molar excess at ambient temperature for 30 min. Excess of biotin was removed by size exclusion chromatography using Zeba™ Spin Desalting Columns 7K MWCO 0.5 mL (Thermo Fisher Scientific,) according to manufacturer's protocol. Analysis of binding kinetics of PF4-specific antibodies in heat-inactivated serum samples or purified IgG fractions was performed using the Octet RED96e system (Sartorius, Goettingen, Germany) as per the manufacturer's recommendations. Data were analysed using the Octet Data Analysis HT 12.0 software applying the 2:1 heterogeneous ligand-binding model. For quantification, the averages of binding between 475 s – 480 s of the association step and 460 s – 462 s of the dissociation step was used to calculate the [%] Residual Binding T_{560s}. The binding profile response of each sample is illustrated as the mean wavelength shift in nm. For details see supplemental material.

Ethics Statement

The study was conducted in accordance with the declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the University of Tuebingen (236/2021BO2, 224/2021BO2).

Statistical analyses

The statistical analysis was performed using GraphPad Prism, Version 8.0 (GraphPad, La Jolla, USA) and violin plots were generated. Comparison between groups was performed by paired or samples *t* test. *P* values <0.05 were considered statistically significant. Data in the text are presented as mean±SEM or n (%).

Data sharing statement

Data may be requested for academic collaborations from the corresponding author.

Results

Patient characteristics

In this study, we used leftover serum samples from 8 patients (4 female, 4 male, median age 38) with suspected thrombotic complications after first vaccination with ChAdOx1 nCoV-19. The mean duration between vaccination and first symptoms was 9 days (range: 5-16 days). All patients had severe thrombocytopenia (mean platelet count: $41x10^9$/L, range, 8 to 60) and increased D-dimer (mean: 29 µg/mL, range, 9 to 54). At admission, thrombotic events were observed in all patients including 6 SVTs and 2 DVT/PE (for more details see Table 1).

IgG from VITT patients induce ex vivo thrombus formation

We investigated the ability of VITT IgG fractions to induce adherent platelets/thrombi using a flow-based microfluidic system. To mimic venous flow conditions, we selected a shear stress of 250 s^{-1} (10 dyne). Our analyses were focused on the role of IgG/platelet interplay. Therefore, platelets from healthy individuals were preincubated with IgGs from VITT patients or healthy controls, added back to autologous whole blood samples and finally perfused through collagen covered microfluidic channels. As shown in figure 1, IgGs from VITT patients caused increased platelet adhesion. Overall, significantly higher surface area coverage by platelets (SAC) was observed in the presence of VITT IgGs in comparison to IgGs from healthy controls (mean % SAC±SEM: 11.59±0.57 vs. 1.99±0.34 respectively,

p<0.001, Figure 1A). Similar findings were also observed when IgG was added directly to whole blood samples. (Supplemental Figure 1).

Inhibition of ex vivo adhesion/thrombus formation by anticoagulants

We further investigated the impact of anticoagulants on SAC by VITT IgGs in our *ex vivo* model. In comparison to untreated controls, SAC was significantly reduced when platelets were pre-incubated with 0.1 and 0.5 U/mL heparin (mean % SAC±SEM: 11.59±0.57 vs.1.85±0.56 and 1.86±0.28 respectively, both p<0.001).

Furthermore, a strong reduction in SAC was observed with danaparoid, as well as argatroban at 0.8 and 8 µg/mL (mean % SAC±SEM: 2.82±0.50, p<0.001; 2.56±0.31 and 1.84±0.66, p<0.001, respectively, Figure 1A). Fondaparinux slightly but significantly reduced SAC at 8 but not 1 µg/mL (mean % SAC±SEM: 7.84±0.701, p=0.01 and 8.82±0.1.6, Figure 1A). Furthermore, the administration of IVIG resulted in inhibition of SAC by VITT IgGs in our microfluidic system in the presence of all anticoagulants including fondaparinux (Mean % SAC±SEM: untreated 12.04±0.75, vs IVIG alone: 4.07±0.51, p<0.001; and IVIG in combination with: heparin 0.1 and 0.5 U/mL 1.71±0.08, and 1.77±0.41 p<0.001; danaparoid 3.55±1.57, p<0.001; fondaparinux 1 and 8 µg/mL 4.25±0.43 and 1.89±1.32, p<0.001; and argatroban 0.8 and 8 µg/mL 2.79±0.48 and 2.44±0.41, p<0.001, figure 1B).

When IgGs from HIT patients were tested, heparin at low concentration had no inhibitory effect on HIT-IgG-induced thrombus formation and rather increased SAC was observed (Supplemental Figure 2).

The effect of anticoagulants on VITT sera-mediated platelet activation

To explore the mechanisms underlying the inhibition of thrombus formation, we thought to investigate the impact of anticoagulants on generation of procoagulant phenotype and platelet aggregation.

VITT sera were incubated with washed platelets in the presence of buffer, varying concentrations of heparin, danaparoid, fondaparinux and argatroban. Flow cytometry

analyses revealed that sera from VITT patients induced significantly higher amount of CD62p/Phosphatidylserine (PS) positive procoagulant platelets in comparison to healthy controls (mean %CD62p/PS positive platelets 40.82±7.02 vs. 0.50±0.16, respectively, p=<0.001, Figure 2A). The generation of procoagulant platelets was reduced by 0.1 U/mL heparin (mean %CD62p/PS positive platelets: 8.04±3.16, p=0.03, Figure 2A). Procoagulant platelet formation was completely inhibited with further increasing concentrations of heparin to 0.5, 1, 5 and 10 U/mL (mean %CD62p/PS positive platelets: 12.59±4.77; 4.41±0.86; 4.71±1.07; and 3.96±1.51, respectively, all p<0.01; Figure 2A). VITT sera-mediated procoagulant platelet formation was also significantly inhibited after incubation with low- and high-dose danaparoid (mean %CD62p/PS positive platelets: 3.03±0.69, p=0.01; and 2.14±0.98, p=0.03, respectively, Figure 2A). Addition of 8 µg/mL Fondaparinux resulted in notable reduction but not complete inhibition of procoagulant platelets (24.36±5.18) but not 0.8 µg/mL (59.85±11.02). On the other hand, treatment of platelets with argatroban had no effect on procoagulant platelets for both the doses tested (mean %CD62p/PS positive platelets: 60.67±12.42 and 38.65±7.73, p>0.05).

To investigate the impact of anticoagulants on platelet aggregation, the HIPA assay was used. Sera were incubated with washed platelets in the presence of buffer (untreated), heparin in different concentrations (0.2, 1, 10 and 100 U/mL), danaparoid, fondaparinux and argatroban. We observed strong platelet activation in presence of buffer in all VITT patients (median time to platelet aggregation: 5 min, range 5-10 min, Figure 2B). However, the reaction was weaker in presence of 0.2 U/mL heparin (median time to aggregate 40-50 min-> no aggregation). All reactions were further inhibited by higher doses of heparin (1, 10 and 100 U/mL, p value= 0.001). Danaparoid also inhibited platelet aggregation but not fondaparinux and argatroban.

Heparin and danaparoid interfere with VITT-IgGs binding to PF4

Next, we intended to understand how anticoagulants interfere with antibody (Ab) function in VITT. To decipher this, PF4 binding to VITT IgGs without any anticoagulants was arbitrarily

set at 100% (baseline). Heparin showed a strong inhibition of PF4 binding to VITT IgGs, and almost 50% inhibition was observed even at a lower concentration of 0.07 U/mL (Figure 3A). Danaparoid also showed a significant inhibition of VITT-IgG binding to PF4, and 50% inhibition was observed at 8.33 U/mL (Figure 3A). However, no differences was observed when fondaparinux or argatroban were added with patients' sera in EIA.

Heparin and danaparoid dissociate VITT-IgGs bound to PF4

Finally, we analyzed the ability of anticoagulants to dissociate VITT-IgGs which are already bound to PF4. VITT Abs were allowed to bind on PF4 coated microtiter plates, before anticoagulants were added. Heparin effectively dissociated the complex, and 75% reduction in IgG binding was observed at a low concentration (0.07 U/mL). Danaparoid also significantly displaced the VITT-Ab/PF4 complex and 50% dissociation was observed starting from 0.26 U/mL. In contrast, fondaparinux and argatroban had no effect on bound VITT-IgGs at all concentrations tested (Figure 3B). Similarly, when VITT sera were incubated with washed platelets from healthy donors in the presence or absence of exogenous PF4, heparin and danaparoid resulted in higher inhibition of IgG binding compared to fondaparinux and argatroban (Figure 3C and 3D).

Binding Kinetics of VITT antibodies

To further investigate the influence of the anticoagulants, particularly on the dissociation of VITT-specific IgGs, we performed biolayer interferometry (BLI) analysis. Therefore, we immobilized biotinylated PF4 on streptavidin biosensors and measured the association of IgGs from serum samples or purified IgG fractions, followed by determining their dissociation in the presence of the various anticoagulants (Figure 4). While binding of PF4 specific antibodies to the biosensor was observed for all VITT samples (n = 5) tested (Figure 4, Supplemental Figure 7 A-F), we found remarkable changes in their dissociation after addition of the various anticoagulants. To highlight differences between anticoagulants, quantification of residual binding at a defined time point (560 s) of dissociation was calculated. In particular, in the presence of heparin (0.1 U/mL, 1 U/mL), we observed increased dissociation of PF4

binding antibodies, resulting in a significant difference in residual binding compared with no anticoagulant (Figure 4C, Supplemental Table 1). A slight but non-significant increase in dissociation was measured for danaparoid, whereas the addition of fondaparinux or argatroban did not appear to affect the dissociation rate. Notably, serum samples and purified IgG fractions showed similar binding kinetics (Supplemental Figure 7A and 7B), confirming that PF4 specific antibody responses were visualized. In comparison, the investigated HIT samples (n=5) applied in the same experimental setting showed high variabilities regarding their dissociation in the presence of the different anticoagulants (Supplemental Figure 7G).

Discussion

In this study, we analyzed the interactions of heparin, danaparoid, fondaparinux and argatroban with VITT-IgG/PF4 complexes. We observed that IgGs from VITT patients are able to induce strong thrombus formation in the absence of anticoagulants. The negatively charged anticoagulants, danaparoid and heparin, were found to be able to interfere with the binding of VITT IgGs to PF4 leading to their dissociation, which in turn inhibits the generation of procoagulant platelets and the subsequent thrombus formation. Moreover, the inhibitory effect of anticoagulants on thrombus formation was more pronounced when IVIG was coadministered. IVIG has been previously shown to inhibit FcγRIIA-mediated activation of platelets by VITT antibodies and also recommended to use in therapy.[8,15]

Heparin is usually preferred as one of the most widely used anticoagulants for prevention and treatment of thrombotic events due to its rapid action, low cost and availability of antidote.[16,17] The use of heparin is, however, discouraged in VITT patients because of clinical similarities observed between VITT and HIT. In contrast to HIT where low concentrations of heparin enhance platelet responses,[18] antibody-mediated platelet activation was inhibited in case of VITT in our study. Recently, successful treatment with heparin was reported in one patient with VITT[19] and in a case series involving three female VITT patients, who developed intracranial venous sinus thrombosis after their first vaccination with ChAdOx1 nCov-19.[20] In a recent study, Smith and colleagues also observed that heparin blocks VITT serum-

mediated platelet aggregation.[9] Recently, VITT antibodies were shown to recognize epitopes within the heparin-binding site on PF4.[21] In fact, VITT antibodies were shown to mimic the effect of heparin by causing PF4 tetramers clustering.[20] In our studies, we found that increasing heparin can disrupt binding of VITT antibodies with PF4 (Figure 3A and 3B). Using BLI, a rapid dissociation of the IgG-PF4 complexes was observed when heparin was added. Other anticoagulants were less effective in disrupting the pre-bound complexes. Due to the polyclonal nature of the antibody pool within serum samples, a calculation of a defined affinity was not possible**.** However, as BLI allows measurement of the association and dissociation of target-specific antibodies, we believe that a clear conclusion about the influence of heparin on the dissociation rate of PF4-specific antibodies could be drawn.

It is interesting, but outside the scope of our current work, to investigate whether heparin blocked the VITT epitopes on PF4 or it induced conformational changes in PF4 that might have reduced the antibody binding-avidity. However, in light of our findings along with other similar recent observations, more studies are warranted to explore the suitability of heparin in VITT patients. Danaparoid is a chemically distinct low-molecular-weight heparinoid devoid of heparin. Danaparoid has different protein-binding properties, making it an excellent candidate for treatment of HIT and prevention of post-operative deep venous thrombosis (DVT).[22,23] In our experimental settings, danaparoid could effectively inhibit VITT sera-mediated platelet responses and thrombus formation, similar to heparin. However, in comparison to heparin, a higher amount of danaparoid was required to inhibit VITT Ab binding to immobilized PF4 in the ELISA (Figure 3A and 3B). Of note, danaparoid inhibited binding of HIT antibodies to PF4 and also disrupted PF4/VITT IgG-complexes (Supplemental Figure 2A and 2B). Our results are in accordance with the findings of Krauel et al., which showed that danaparoid inhibits binding of anti-PF4/heparin antibodies to PF4/heparin complexes up to 30% at prophylactic doses and 50% at therapeutic doses.[24] Our data indicate that, in addition to its anticoagulatory effects, danaparoid can also interfere with antibody-mediated platelet activation and might protect against new thrombotic events in

patients with VITT. The prompt onset of action might be another advantage of this anticoagulant during acute VITT due to the ability to disassociate PF4/VITT-IgG complexes.

Another interesting finding in our study was that argatroban showed no direct effect on the formation of procoagulant platelets, platelet activation and disruption of VITT Ab binding to PF4 but significantly inhibited the *ex vivo* thrombus formation. Fondaparinux, a selective factor Xa inhibitor, is another non-heparin anticoagulant that is used in patients with HIT[25] and also recommended in the treatment of VITT.[26] Among non-heparin anticoagulants, fondaparinux has been shown to have the lowest thrombotic risk and all-cause-mortality rate in HIT.[25] Fondaparinux showed slight inhibition of *ex vivo* thrombus formation, which was significantly increased when IVIG was coadminstrated. While fondaparinux reduced the formation of procoagulant platelets and thrombus formation, it failed to interfere with the formation of immune complexes from PF4 and VITT or HIT antibodies (Figure 3A, Supplemental Figure 4A). Similar to our observations, Krauel *et al.* found that fondaparinux at therapeutic concentrations does not inhibit antibody binding to PF4/heparin complexes and platelet activation in serotonin release assay in HIT.[23] The differences between these polyanions might be due to different degrees of sulfation. The latter was shown to be responsible for structural changes in PF4 resulting in antiparallel β-sheet content >30% that makes PF4/polyanion complexes for example antigenic for the HIT antibodies.[27-29] While our data provide a new insight on the interaction between anticoagulants and VITT-PF4 immune complexes, clinical studies are needed to investigate whether anticoagulants that showed higher inhibition of thrombus formation *ex vivo* might be more efficient to treat patients with VITT.

Nevertheless, our studies show that heparin and danaparoid interfere with *in vitro* antibody-binding to PF4 and platelets leading to sufficient inhibition of platelet activation and generation of procoagulant phenotype. In the absence of the procoagulant platelet phenotype, no thrombus was observed in our *ex vivo* model. This finding indicates that antibody-mediated generation of procoagulant platelets is essential for the development of thrombosis in VITT. On the other hand, thrombin inhibition prevented the thrombus formation

despite the lack of impact on antibody binding and subsequent platelet activation. These data suggest that thrombus formation in our *ex vivo* model is dependent on thrombin. In contrast to argatroban, heparin and danaparoid might inhibit VITT-induced thrombosis by interfering with three steps; antibody-binding, platelet activation/generation of procoagulant phenotype and factor X activation, while fondaparniux seems to exist mainly on anti-Xa activity.

While our *ex vivo* microfluidic model might provide preclinical information on the performance of therapeutic approaches to thrombosis, further investigations on role of complement activation in VITT are needed to understand the disease pathophysiology and better management. *In vivo* animal models, when available, will be able to show the capacity of the VITT antibodies to induce thrombosis and the ability of anticoagulants to block it. In addition, the influence of other cell types (e.g. endothelial cells) and other *in vivo* factors (e.g. distribution of the drug within the animal's body and binding of the drug to tissues) can only be addressed *in vivo*.

In conclusion, our study suggests that heparin and danaparoid can inhibit thrombus formation in VITT patients in part through the inhibition of the interaction of VITT antibodies with PF4 and subsequent platelet activation. The effect of argatroban is independent from the interaction of VITT antibodies with PF4. To our best knowledge, this is the first study to investigate systematically the interactions of heparin and non-heparin anticoagulants with VITT-Ab/PF4 complexes. Our results should be confirmed, however, in a clinical setting before a recommendation regarding the selection of anticoagulation in VITT patients could be made.

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Authorship contributions

A.S. and T.B. designed the study. A.S., P.T., T.R.W., L.P., J.Z., V.F. and K.W. performed the experiments. G.U., K.A., S.N-H. and T.B. provided clinical data. A.S., P.T., G.U., T.R.W., U.R., K.A. and T.B. analyzed the data, interpreted the results and wrote the manuscript. All authors read and approved the manuscript prior to submission.

Conflict of interest disclosures

Tamam Bakchoul has received research funding from CoaChrom Diagnostica GmbH, DFG, Robert Bosch GmbH, Stiftung Transfusionsmedizin und Immunhämatologie e.V.: Ergomed, Surrey, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden-Wuerttembergm, has received lecture honoraria from Aspen Germany GmbH, Bayer Vital GmbH, Bristol-Myers Squibb GmbH & Co., Doctrina Med AG, Meet The Experts Academy UG, Schoechl medical education GmbH, Mattsee, Stago GmbH, Mitsubishi Tanabe Pharma GmbH, Novo Nordisk Pharma GmbH, has provided consulting services to: Terumo, has provided expert witness testimony relating to heparin induced thrombocytopenia (HIT) and non-HIT thrombocytopenic and coagulopathic disorders. All of these are outside the current work. Other authors declare no competing financial interests.

References

1. Callaway E, Ledford H, Viglione G, Watson T, Witze A. COVID and 2020: An extraordinary year for science. *Nature*. 2020;588(7839):550-552.
2. COVID research: a year of scientific milestones. *Nature*. 2021.

3. See I, Su JR, Lale A, et al. US Case Reports of Cerebral Venous Sinus Thrombosis With Thrombocytopenia After Ad26.COV2.S Vaccination, March 2 to April 21, 2021. *JAMA*. 2021;325(24):2448-2456.
4. Schultz NH, Sorvoll IH, Michelsen AE, et al. Thrombosis and Thrombocytopenia after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. 2021;384(22):2124-2130.
5. Greinacher A, Thiele T, Warkentin TE, Weisser K, Kyrle PA, Eichinger S. Thrombotic Thrombocytopenia after ChAdOx1 nCov-19 Vaccination. *N Engl J Med*. 2021;384(22):2092-2101.
6. Althaus K, Moller P, Uzun G, et al. Antibody-mediated procoagulant platelets in SARS-CoV-2-vaccination associated immune thrombotic thrombocytopenia. *Haematologica*. 2021;106(8):2170-2179.
7. Scully M, Singh D, Lown R, et al. Pathologic Antibodies to Platelet Factor 4 after ChAdOx1 nCoV-19 Vaccination. *N Engl J Med*. 2021;384(23):2202-2211.
8. Uzun G, Althaus K, Singh A, et al. The use of intravenous immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia. *Blood*. 2021.
9. Smith CW, Montague SJ, Kardeby C, et al. Anti-Platelet Drugs Block Platelet Activation by Vaccine-Induced Immune Thrombocytopenia and Thrombosis Patient Serum. *Blood*. 2021.
10. Oldenburg J, Klamroth R, Langer F, et al. Diagnosis and Management of Vaccine-Related Thrombosis following AstraZeneca COVID-19 Vaccination: Guidance Statement from the GTH. *Hamostaseologie*. 2021;41(3):184-189.
11. Nazy I, Sachs UJ, Arnold DM, et al. Recommendations for the clinical and laboratory diagnosis of VITT against COVID-19: Communication from the ISTH SSC Subcommittee on Platelet Immunology. *J Thromb Haemost*. 2021;19(6):1585-1588.
12. Roest M, Reininger A, Zwaginga JJ, King MR, Heemskerk JW, Biorheology Subcommittee of the SSCotI. Flow chamber-based assays to measure thrombus formation in vitro: requirements for standardization. *J Thromb Haemost*. 2011;9(11):2322-2324.
13. Herfs L, Swieringa F, Jooss N, et al. Multiparameter microfluidics assay of thrombus formation reveals increased sensitivity to contraction and antiplatelet agents at physiological temperature. *Thromb Res*. 2021;203:46-56.
14. Krauel K, Hackbarth C, Furll B, Greinacher A. Heparin-induced thrombocytopenia: in vitro studies on the interaction of dabigatran, rivaroxaban, and low-sulfated heparin, with platelet factor 4 and anti-PF4/heparin antibodies. *Blood*. 2012;119(5):1248-1255.
15. Rizk JG, Gupta A, Sardar P, et al. Clinical Characteristics and Pharmacological Management of COVID-19 Vaccine-Induced Immune Thrombotic Thrombocytopenia With Cerebral Venous Sinus Thrombosis: A Review. *JAMA Cardiol*. 2021.
16. Calderón-Gerstein W. Prophylaxis for thromboembolism in hospitalized medical patients. *N Engl J Med*. 2007;357(3):304-305; author reply 305-306.
17. Piran S, Schulman S. Treatment of bleeding complications in patients on anticoagulant therapy. *Blood*. 2019;133(5):425-435.
18. Vayne C, Guery EA, Kizlik-Masson C, et al. Beneficial effect of exogenous platelet factor 4 for detecting pathogenic heparin-induced thrombocytopenia antibodies. *Br J Haematol*. 2017;179(5):811-819.
19. Tiede A, Sachs UJ, Czwalinna A, et al. Prothrombotic immune thrombocytopenia after COVID-19 vaccine. *Blood*. 2021.
20. Wolf ME, Luz B, Niehaus L, Bhogal P, Bazner H, Henkes H. Thrombocytopenia and Intracranial Venous Sinus Thrombosis after "COVID-19 Vaccine AstraZeneca" Exposure. *J Clin Med*. 2021;10(8).
21. Huynh A, Kelton JG, Arnold DM, Daka M, Nazy I. Antibody epitopes in vaccine-induced immune thrombotic thrombocytopaenia. *Nature*. 2021.
22. Chong BH, Gallus AS, Cade JF, et al. Prospective randomised open-label comparison of danaparoid with dextran 70 in the treatment of heparin-induced thrombocytopaenia with thrombosis: a clinical outcome study. *Thromb Haemost*. 2001;86(5):1170-1175.
23. Farner B, Eichler P, Kroll H, Greinacher A. A comparison of danaparoid and lepirudin in heparin-induced thrombocytopenia. *Thromb Haemost*. 2001;85(6):950-957.

24. Krauel K, Furll B, Warkentin TE, et al. Heparin-induced thrombocytopenia--therapeutic concentrations of danaparoid, unlike fondaparinux and direct thrombin inhibitors, inhibit formation of platelet factor 4-heparin complexes. *J Thromb Haemost*. 2008;6(12):2160-2167.
25. Nilius H, Kaufmann J, Cuker A, Nagler M. Comparative effectiveness and safety of anticoagulants for the treatment of heparin-induced thrombocytopenia. *Am J Hematol*. 2021;96(7):805-815.
26. Furie KL, Cushman M, Elkind MSV, Lyden PD, Saposnik G. Diagnosis and Management of Cerebral Venous Sinus Thrombosis With Vaccine-Induced Immune Thrombotic Thrombocytopenia. *Stroke*. 2021;52(7):2478-2482.
27. Brandt S, Krauel K, Gottschalk KE, et al. Characterisation of the conformational changes in platelet factor 4 induced by polyanions: towards in vitro prediction of antigenicity. *Thromb Haemost*. 2014;112(1):53-64.
28. Brandt S, Krauel K, Jaax M, et al. Polyphosphates form antigenic complexes with platelet factor 4 (PF4) and enhance PF4-binding to bacteria. *Thromb Haemost*. 2015;114(6):1189-1198.
29. Cines DB, Yarovoi SV, Zaitsev SV, et al. Polyphosphate/platelet factor 4 complexes can mediate heparin-independent platelet activation in heparin-induced thrombocytopenia. *Blood Adv*. 2016;1(1):62-74.

Figure Legends

Figure 1

Impact of anticoagulants on VITT IgG-mediated platelet adhesion/thrombus formation

A) Platelets from healthy individuals were incubated with IgGs from healthy controls (HC) or VITT patients prior to calcein staining and reconstitution with autologous whole blood, and perfusion through BioFlux microfluidic channels. IgGs concentrations were measured using nanodrop (Mean IgG conc. 15.10±2.14 mg/mL). Where indicated, samples were treated with different concentrations of anticoagulants: low-dose heparin (LD heparin, 0.1 U/mL), therapeutic-dose heparin (TD heparin, 0.5U/mL), TD danaparoid (0.8 U/mL), TD and HD fondaparinux (1 and 8 µg/mL) and TD and HD of argatroban (0.8 and 8 µg/mL).

B) IVIG was added alone or in combination with anticoagulants. Each dot represents one VITT patient IgG. Images were acquired at x20 magnification using an inverted Olympus IX73 or Zeiss Axio Observer 7 fluorescence microscope. Thrombus formation was determined as the percent of total surface area coverage (%SAC) by adherent platelets using ImageJ. Images were processed identically using automated or manually adjusted threshold settings, and exclusion of image artefacts. Violin plots showing distribution of the values were generated using Graphpad Prism 8. Abbreviations: LD: Low dose; TD:

Therapeutic dose; HD: High dose; ns= not significant; *p<0.05, **p<0.01 and ***p<0.001. This figure represents values from same experimental set up. Figure is split in 2 different panels (1A and 1B) for easier interepretation of results. Therefore values and representative images in HC (healthy controls) group and some in untreated VITT IgGs are presented in both panels.

Figure 2

VITT IgG-mediated procoagulant platelets and platelet activation in the presence of anticoagulants

A) Procoagulant platelets (CD62P/Phosphatidylserine (PS) positive cells were analyzed with flow cytometry after incubation of healthy platelets with VITT sera or sera from healthy controls (HC) in different settings, and staining with Annexin V-FITC and CD62p-APC antibody. Where indicated, platelets were co-incubated with different concentrations of anticoagulants: heparin (unfractionated heparin, 0.1, 0.5, 1, 5 and 10 U/mL), danaparoid (0.8 and 8 U/mL), fondaparinux (1 and 8 µg/mL) and argatroban (0.8 and 8 µg/mL). Perecent (%) of CD62p/PS double positive platelets is shown as violin plots. Each dot represents sera from an individual VITT patient, and number of sera tested is reported in each graphic. Violin plots showing distribution of the values were generated using Graphpad Prism 8. Abbreviations: ns = not significant, *p<0.05, **p<0.01, ***p<0.001 and ***p<0.0001.

B) Results of the platelet activation assay (HIPA). Each dot represents the median value of two different healthy platelet donors. VITT patients showed strong platelet activation with buffer alone in comparison to healthy controls (HC), which was inhibited with varying concentration of heparin. Where indicated, platelets were treated with different concentrations of anticoagulants: heparin (unfractionated heparin, 0.2, 1, 10 and 100 U/mL), danaparoid (0.8 U/mL), fondaparinux (8 µg/mL) and argatroban (8 µg/mL). Data are presented as time to aggregate. Violin plots showing distribution of the values were generated using Graphpad Prism 8. Abbreviations: ns = not significant, *p<0.05 and **p<0.01.

Figure 3

Heparin and danaparoid, but not fondaparinux or argatroban interfere with anti-PF4 Ab binding to PF4

A) Sera from VITT patients with anti-PF4 IgG were tested in an in-house PF4 ELISA. Sera were incubated on PF4 (25 µg/mL) coated microplates. Where indicated, increasing concentrations (0.07, 0.13, 0.26, 0.52, 1.04, 2.08, 4.17, 8.33, 16.67, and 33.33) of UFH and danaparoid (both U/mL) or fondaparinux and argatroban (both µg/mL) were added as described previously.[14] Heparin and danaparoid equally reduced the Ab binding to PF4. However, fondaparinux and argatroban had no effect. Each dot represents mean value of VITT patients' sera tested, and the numbers are reported in each graphic for each anticoagulant. (N=3-6). The signal of anti-PF4 Ab binding without anticoagulants was set to 100%. Data are shown as mean ± SEM.

B) Sera from VITT patients with anti-PF4 IgG were tested in an in-house PF4 ELISA to analyze the VITT-IgG/PF4 complex dissociation by anticoagulants. Patient sera were first incubated in PF4 coated microplates for one hour to facilitate VITT-IgG/PF4 binding and complex formation. Subsequently increasing amounts (0.07 to 33.33 U or µg/mL) of anticoagulants were added on VITT-IgG/PF4 complex containing microplates. While heparin and danaparoid potently disrupted the IgGs bound to PF4, no disruption was observed with fondaparinux and argatroban. Each dot represents mean value of VITT patients' sera tested, and the numbers are reported in each graphic for each anticoagulant. (N=3). The signal of anti-PF4 Ab binding without anticoagulants was set to 100%. Data are shown as mean ± SEM.

C) Immunoglobulin G (IgG) binding to healthy washed platelets after incubation with sera from VITT patients in presence of PF4 (25 µg/mL) or without PF4 (D) was assessed by flow cytometry and expressed as fold increase (FI) normalized to healthy controls (HC). VITT patients showed significantly higher binding at the baseline in comparison to healthy controls, which was significantly inhibited by heparin and danaparoid. Fondaparinux and Argatroban

also showed slight displacement of IgG binding. Violin plots showing distribution of the values were generated using Graphpad Prism 8. ns: not significant; *P<0.05, and **P<0.01.

Figure 4

Binding kinetics of PF4 specific antibodies from VITT samples in presence of different anticoagulants.

Biotinylated PF4 (5 µg/mL) was immobilized on streptavidin biosensor tips and binding kinetics of serum samples from patients with VITT (n = 5) were analyzed using biolayer interferometry (BLI). For dissociation, different anticoagulants [heparin 0.1 U /mL (light red), heparin 1 U/ mL (dark red), danaparoid 0.8 U/ mL, fondaparinux 8 µg/ mL, argatroban 8 µg/ mL] or Octet buffer were applied. Curves and data points represent the mean of technical duplicates (n = 2).

A) Schematic illustration of the experimental setup.

B) Representative binding responses of PF4 specific antibodies from a VITT patient serum (VITT1).

C) Quantification of residual binding in percent at time point 560s of the dissociation ([%] Residual Binding T560s) of VITT serum samples.

Table 1: Patient characteristics

	sex	First symptoms after vaccination (days)	Thrombosis	Platelet count	D-Dimer	PF4-Heparin-EIA (OD)	Platelet activation with PF4 (modified HIPA)
#1	m	16	SVT/DVT/PE	60	12	3.4	+
#2	m	8	SVT	22	n.a	3.1	+
#3	f	6	SVT	40	n.a	2.2	+
#4	f	9	SVT	27	54	3.2	+
#5	f	7	SVT	53	35	3.1	+
#6	f	5	SVT	56	9	2.1	+
#7	m	9	DVT/PE	8	>35	3.2	+
#8	m	12	DVT/PE	60	n.a.	3.0	+

m=male, f=female, SVT=sinus vein thrombosis, DVT=deep vein thrombosis, PE=pulmonary embolism, OD=optical density, PF4=platelet factor 4, HIPA=Heparin induced platelet activation assay

Figure 1

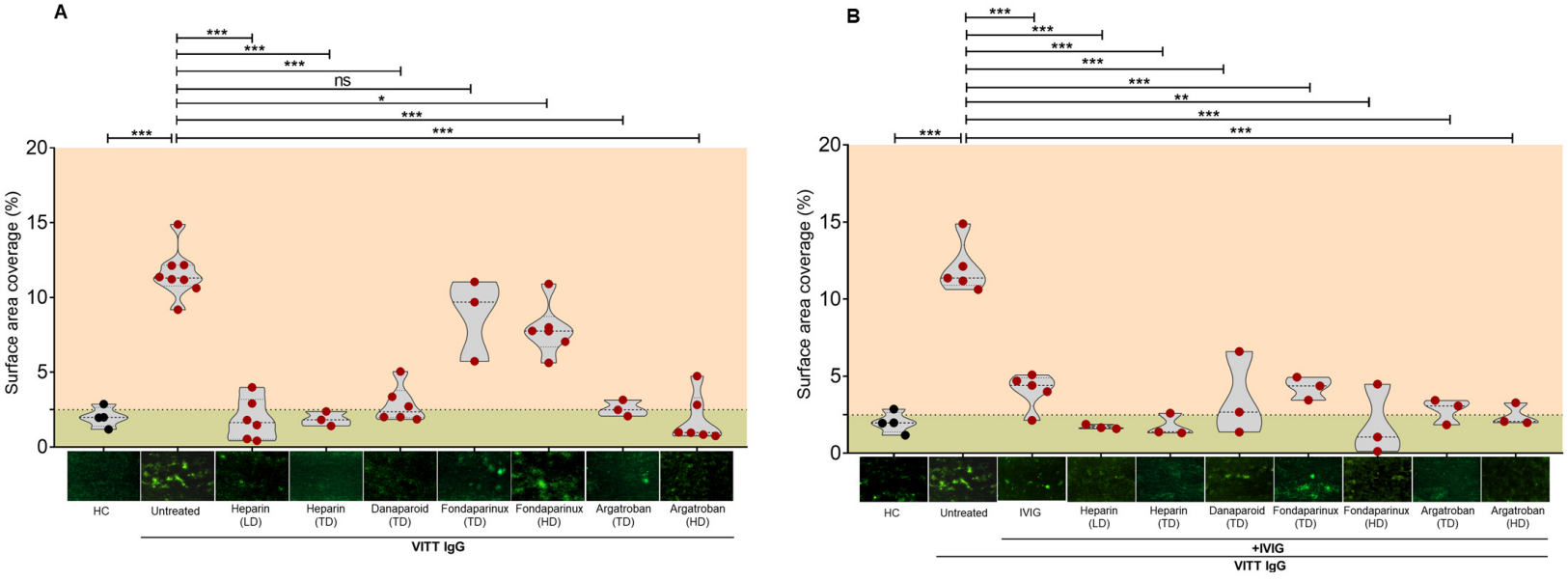

A
B
Surface area coverage (%)
0
5
10
15
20
***
ns
*
**
HC
Untreated
IVIG
Heparin (LD)
Heparin (TD)
Danaparoid (TD)
Fondaparinux (TD)
Fondaparinux (HD)
Argatroban (TD)
Argatroban (HD)
+IVIG
VITT IgG

Figure 2

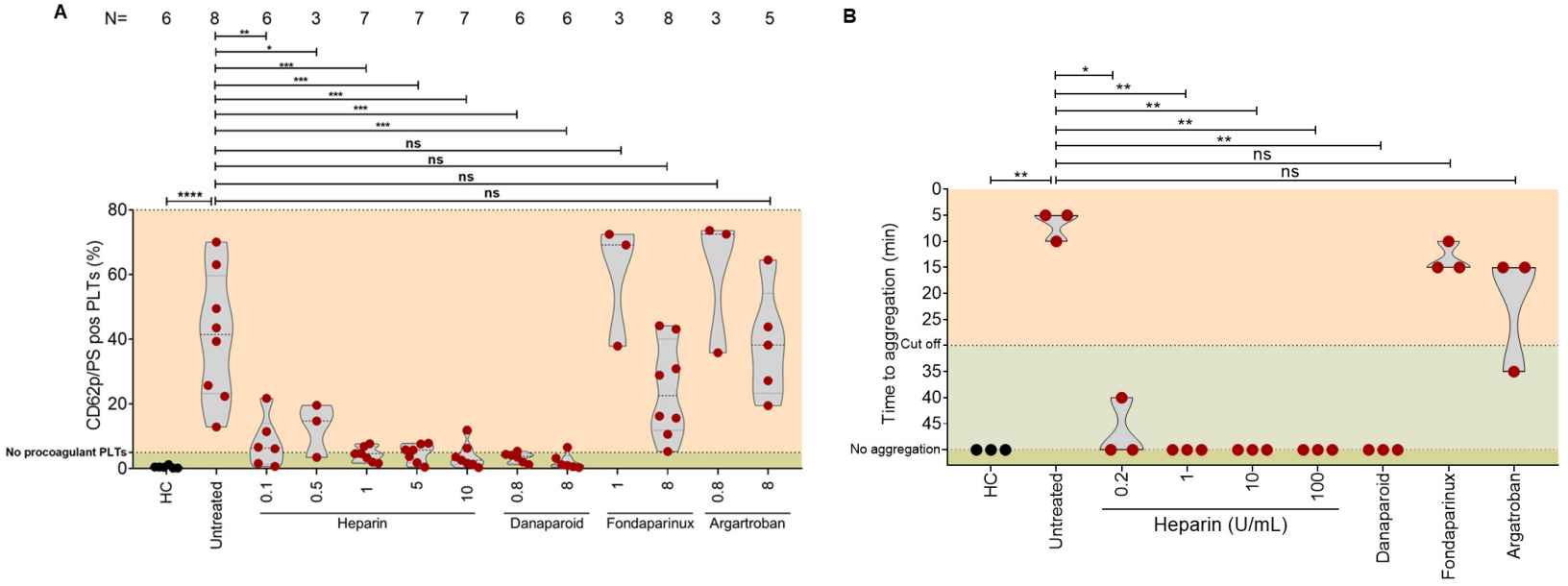

A
N= 6 8 6 3 7 7 7 6 6 3 8 3 5
CD62p/PS pos PLTs (%)
No procoagulant PLTs
HC
Untreated
0.1
0.5
1
5
10
0.8
8
1
8
0.8
8
Heparin
Danaparoid
Fondaparinux
Argartroban
B
Time to aggregation (min)
Cut off
No aggregation
HC
Untreated
0.2
1
10
100
Heparin (U/mL)
Danaparoid
Fondaparinux
Argatroban

Figure 3

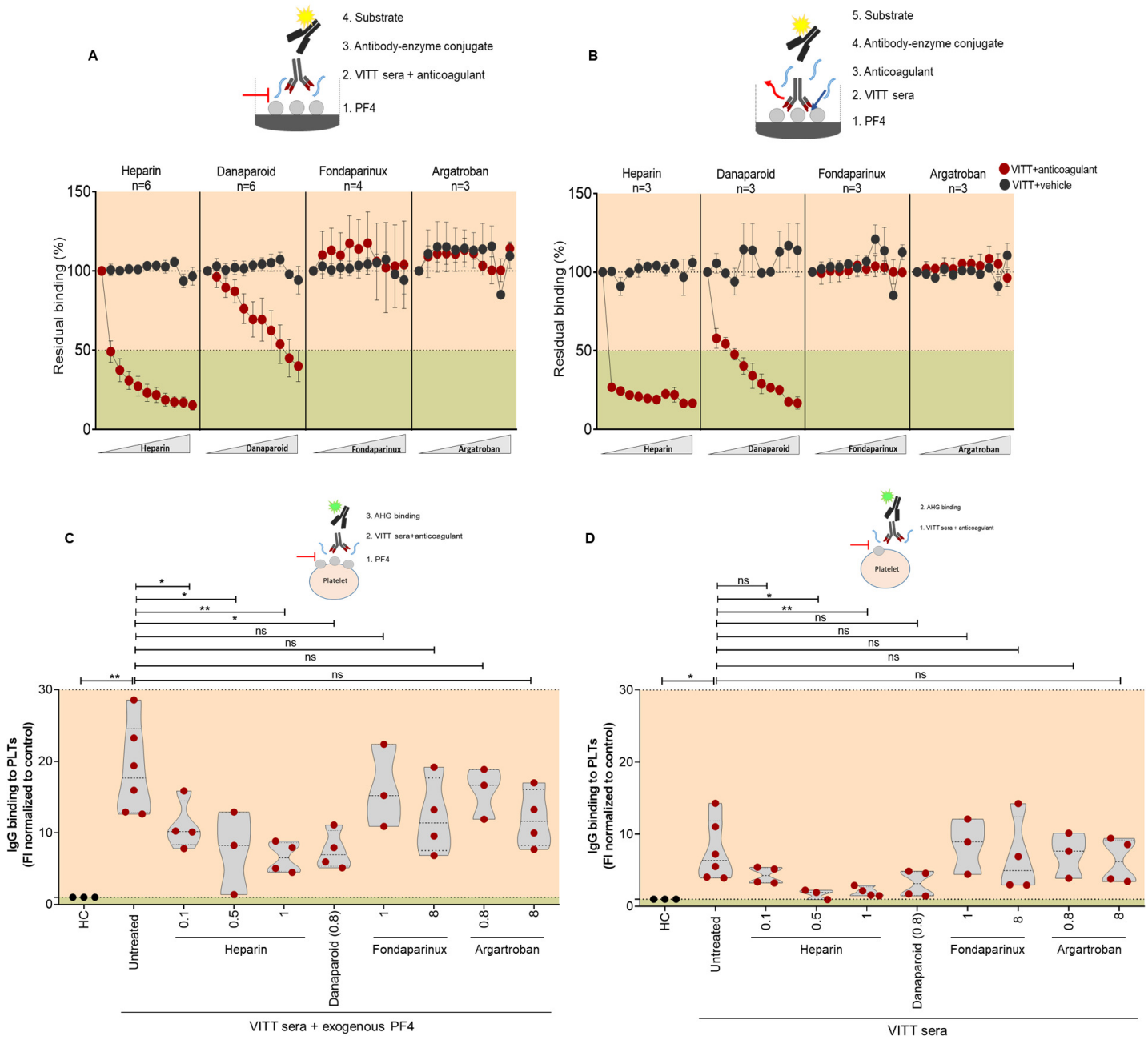
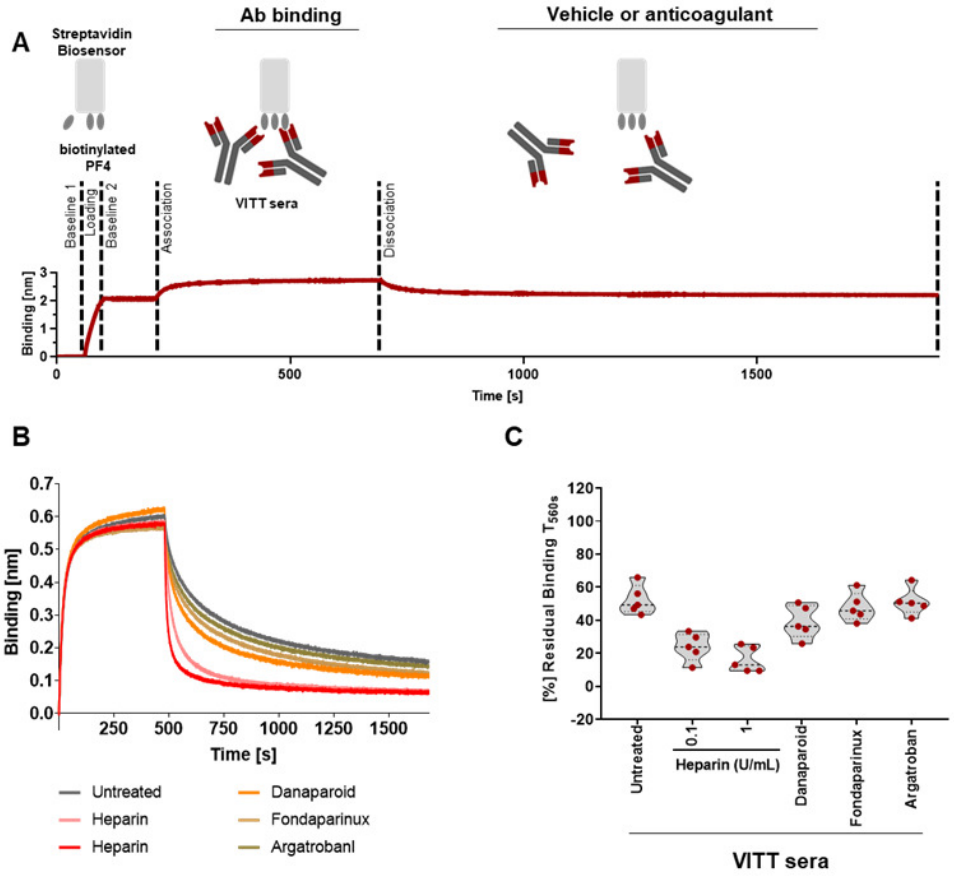

A
4. Substrate
3. Antibody-enzyme conjugate
2. VITT sera + anticoagulant
1. PF4
B
5. Substrate
4. Antibody-enzyme conjugate
3. Anticoagulant
2. VITT sera
1. PF4
Heparin n=6
Danaparoid n=6
Fondaparinux n=4
Argatroban n=3
Heparin n=3
Danaparoid n=3
Fondaparinux n=3
Argatroban n=3
VITT+anticoagulant
VITT+vehicle
Residual binding (%)
150
100
50
0
Heparin
Danaparoid
Fondaparinux
Argatroban
C
3. AHG binding
2. VITT sera+anticoagulant
1. PF4
Platelet
D
2. AHG binding
1. VITT sera + anticoagulant
Platelet
IgG binding to PLTs
(FI normalized to control)
30
20
10
0
HC
Untreated
0.1
0.5
1
Heparin
Danaparoid (0.8)
1
8
Fondaparinux
0.8
8
Argartroban
VITT sera + exogenous PF4
VITT sera
ns
*
**

Figure 4


A
Streptavidin Biosensor
biotinylated PF4
Baseline 1
Loading
Baseline 2
Association
VITT sera
Dissociation
Ab binding
Vehicle or anticoagulant
Binding [nm]
Time [s]
B
Binding [nm]
Time [s]
Untreated
Heparin
Heparin
Danaparoid
Fondaparinux
Argatroban
C
[%] Residual Binding T560s
Untreated
0.1
1
Heparin (U/mL)
Danaparoid
Fondaparinux
Argatroban
VITT sera